

Angiogenesis Imaging Methodology: AIM for Clinical Trials
The Role of Imaging in Clinical Trials of Anti-Angiogenesis Therapy in Oncology

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Oncology clinical trials frequently use anatomical imaging to provide indices of therapeutic response. Most trials employ simple linear measurements to estimate changes in tumor mass in response to the investigational therapy as compared with a baseline measure. Response criteria are pre-established by the protocol to define some expected percent change in one or a combination of measurements for classification of response, stable disease, or progression. There is considerable controversy over the adequacy of these measurements and subsequent definition of response criteria, and recently there have been attempts at standardization (RECIST). Although this is an important step forward, it is overshadowed by the new challenges that have resulted from the widespread inclusion of functional imaging in clinical trials of new targeted therapies.

These challenges are neither trivial nor remote and are currently being faced in the growing number of clinical trials related to angiogenesis, particularly in the evaluation of therapeutic response to angiostatin. Because effective angiostatin therapy may not lead to substantial tumor mass/volume reduction, especially soon after therapy, conventional measurements of response may be insensitive or markedly delayed even when there is a significant therapeutic effect. Interest in imaging techniques that can provide an early indicator of effectiveness at a functional or molecular level has therefore increased. Current clinical trials employ a wide range of imaging techniques, including PET (especially with FDG), MRI, ultrasound and occasionally CT, in an attempt to evaluate changes in blood volume, blood flow alterations, permeability alterations, and in the case of PET changes in glucose metabolism (FDG). It is unclear whether any of these techniques can distinguish new vascular growth from existing tumor vascularity or, most importantly, provide a quantitative measure of significant reduction in tumor vascular growth.

In an attempt to define the status of currently available clinical imaging and to determine the potential of imaging technology at the translational stage of development, the Biomedical Imaging Program of the National Cancer Institute sponsored "Angiogenesis Imaging Methodology: AIM for Clinical Trials," a workshop held February 26-27, 2000 in Incline Village, Nevada. The workshop brought together experts from four imaging modalities (Ultrasound, CT, PET/Nuclear, MRI) to discuss the current and future role of imaging in clinical trials of anti-angiogenic therapy in oncology. Each modality team prepared extensive background reports that were presented and discussed at the meeting. This series of publications comprises the final reports, reviews from the two keynote speakers, and the recommendations of the teams, and presents the current status and future potential of imaging in the detection and evaluation of angiogenesis.

This WEB submission includes the entirety of one of the keynote overview presentations and the recommendation sections of each modality panel. *Academic Radiology* will publish the complete set of documents including both keynote submissions and the extensive reviews generated by each modality panel in a special addition later this year.

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The following individuals participated in the workshop:

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TUMOR ANGIOGENESIS: MOLECULAR PATHOLOGY, THERAPEUTIC TARGETING, AND IMAGING

William W. Li, MD

INTRODUCTION

Angiogenesis, the growth of new blood vessels, is a fundamental physiological process required for development, reproduction, wound repair, and response to ischemia.¹ Pathological angiogenesis, often referred to as neovascularization, is associated with disease conditions, including retinopathies, arthritis, psoriasis, and cancer.²⁻⁵ Folkman first hypothesized in 1971 that solid tumors remain growth restricted to 2-3 mm in diameter until the onset of angiogenesis.⁶ Subsequent investigations have broadened the study of angiogenesis into a distinct field of scientific inquiry that has yielded insights into vascular growth regulation, including: the identification, sequencing and cloning of at least 20 angiogenic growth factors, their receptors and signal transduction pathways; the discovery of endogenous angiogenesis inhibitors; and the cellular and molecular characterization of the angiogenic phenotype in human cancers. (For reviews, see references 7 and 8.)

One practical dividend from angiogenesis research is the large-scale biopharmaceutical industry effort to develop new angiogenesis-modulating drugs. Angiogenesis-dependent diseases afflict as many as 500 million patients in western nations each year.⁹ Both pro- and anti-angiogenic drugs are in advanced stages of clinical development. "Antiangiogenesis" is an emerging therapeutic strategy in clinical oncology aimed at halting cancer progression by suppressing the tumor blood supply.⁶ A large body of preclinical animal research, and individual case reports in cancer patients support the validity of antiangiogenic therapy.¹⁰⁻¹⁴ In the US, Canada, Europe, and Australia, there are more than three dozen experimental antiangiogenic agents currently in use in human clinical trials aimed at a broad range of solid tumors, multiple myeloma, leukemia, and lymphoma.¹⁵⁻¹⁷ To date, an estimated \$4 billion has been invested by the public and private sectors to develop such agents, making this one of the most heavily invested areas of cancer research in human history.

This paper reviews the molecular pathology of tumor angiogenesis, surveys the therapeutic targets considered attractive for antiangiogenic drug development, and outlines the new paradigms for cancer detection, intervention, and imaging that are emerging. Finally, unique characteristics of tumor angiogenesis that may be exploited for evaluating the angiogenic process are discussed.

THE MOLECULAR PATHOLOGY OF ANGIOGENESIS IN TUMOR GROWTH & PROGRESSION

Early tumor interactions with the vasculature

All solid tumors begin their existence as small populations of transformed cells whose growth is governed by a balance between tumor cell proliferation and apoptosis.¹⁸ Because early tumors lack an independent blood supply, tumor expansion is restricted by the lack of access to circulating oxygen, nutrients and growth factors. To overcome these limitations, early tumors

grow directionally towards pre-existing nearby blood vessels, a process termed 'vessel cooption.'¹⁹ Tumor cells may infiltrate these blood vessels regionally to form a 'mosaic' vessel consisting of normal vascular endothelial cells interspersed with infiltrative tumor cells. Such changes may occur when the tumor mass is comprised of only 60-80 cancer cells.²⁰ Vascular cooption serves only the tumor periphery, however, so gradual tumor expansion leads to increasing central hypoxia. Hypoxia induces gene expression of the angiogenic growth factor, vascular endothelial growth factor, through hypoxia-inducible factor- α , a phenomenon also observed in ischemic cardiovascular and ocular tissues.^{4,21-23} This initial phase of limited tumor growth may persist for months or even years.

Switch to the angiogenic phenotype enables tumor progression

A rapid phase of tumor growth occurs when the tumor switches to its angiogenic phenotype. This process has been extensively studied in a transgenic mouse model of multi-stage pancreatic islet cell carcinogenesis (RIP1-Tag2), where clonal expansion of a subset of hyperplastic cells secreting angiogenic growth factors leads to frank tumor progression.^{24,25} Both human and experimental cancers produce numerous peptide angiogenic factors, although vascular endothelial growth factor (VEGF), acidic and basic fibroblast growth factors (aFGF, bFGF), and platelet-derived endothelial cell growth factor (PD-ECGF) are perhaps the best studied (Table 1). The production of angiogenic factors by tumors is profound and sustained. In human cancers, factors such as VEGF, bFGF, TGF- β , TNF- α , and PD-ECGF can be detected *in situ* within tumor specimens, circulating in serum, plasma and cerebrospinal fluid, and excreted in the urine.²⁶⁻²⁹ In addition to hypoxia, other factors in the tumor microenvironment, such as acidosis and inflammation, may amplify angiogenic factor expression.³⁰⁻³²

Once angiogenesis is initiated, tumors expand exponentially and invade tissues locally. Tumor cells secrete angiogenic factors that promote new blood vessel growth, while vascular endothelial cells provide oxygen and paracrine growth factors that drive tumor cell growth.³³ Thus, a virtual cycle sustains both cellular compartments.² Neovascular channels allow tumor cells to metastasize hematogenously. For example, ten to 100 million endothelial cells are required to support the smallest palpable breast cancer, one centimeter in diameter, which weighs one gram and consists of approximately one billion cancer cells.³⁴ For a tumor this size, 2×10^6 cancer cells are shed into the systemic circulation every 24 hours.³⁵ Of these, only one cancer cell survives and lodges in a distant organ as a micrometastases, remaining quiescent until the tumor angiogenesis occurs in that site.

Establishment of the angiogenic phenotype requires tumors to produce angiogenic factors in excess of local angiogenesis inhibitors.³⁶ The first endogenous tissue angiogenesis inhibitor to be associated with negative tumor regulation was thrombospondin-1.³⁷ Since then, numerous endogenous inhibitors have been identified, including angiostatin, endostatin, antiangiogenic antithrombin III, pigment epithelium-derived factor, 2-methoxy-estradiol, vasostatin, canstatin and some 20 others.³⁸⁻⁴⁰ The phenomenon of concomitant tumor resistance, in which removal of a primary tumor leads to growth of metastases, has been attributed to the production by primary tumors of circulating antiangiogenic molecules, such as angiostatin and endostatin.^{2,18} While the mechanisms of action of many endogenous inhibitors remains poorly defined, inhibition of endothelial cell migration, proliferation and induction of endothelial cell apoptosis have all been described.^{41,42}

The angiogenic cascade

Angiogenic growth factors elaborated by tumor cells and tumor-infiltrating inflammatory cells initiate a well-characterized cascade of cellular and molecular events:

Angiogenic growth factor production and release

Angiogenic growth factors diffuse towards nearby pre-existing blood vessels (venules and capillaries) and bind to unique receptors located on endothelial cells and their precursors. Among the best studied are the cognate receptors of VEGF (VEGFR-1/Flt-1, VEGFR-2/KDR/Flk-1, VEGFR-3/KDR, Flt-1, VEGF-R2, VEGF-R3/Flt-4, VEGF-R4/neuropilin-1).²² Participating cells may be derived from adult endothelium or circulating endothelial progenitor cells.⁴³

Endothelial receptor binding and activation

Growth factor ligand-receptor binding initiates receptor dimerization and activation of multiple signal transduction pathways, including phosphorylation of tyrosine kinases, protein kinases, and MAP kinases, leading to endothelial gene expression and cell proliferation.⁴⁴⁻⁴⁶ VEGF and bFGF ligand-receptor binding also leads to expression of antiapoptotic molecules, such as Bcl-2 and survivin, suggesting that certain growth factors promote endothelial cell survival.^{47,48} The angiogenic mediator angiopoietin-1 promotes antiapoptosis through phosphorylation of the Tie-2 receptor and the phosphatidylinositol 3'-kinase/Akt signal transduction pathway.⁴⁹

Formation of angiogenic mother vessels

Once endothelial cells become activated, the parent vessel undergoes a discrete morphological change, enlarging in cross-sectional area by 3-to-4 fold to form a 'mother' vessel originating from capillaries or venules.⁵⁰ Mother vessels are characterized by a thinned endothelial cell lining, increased endothelial number, decreased pericyte numbers, pericyte detachment, and basement membrane degradation. In the setting of VEGF, mother vessels are hyperpermeable, with increased numbers of fenestrae and prominent collections of vesiculo-vacuolar organelles (VVO), compared to normal microvessels.⁵¹ Hence, some of the earliest histopathological features of angiogenesis are local microvascular dilatation, hyperpermeability, extravascular fibrin deposition and edema.

Morphogenesis of mother vessels

Newly formed mother vessels are transient, lasting only days. They then undergo at least four divergent morphological pathways: i) muscular artery/vein formation (occurring in 1-3 months); ii) vascular bridging (occurring in 3 days - 3 weeks); iii) intussusceptive microvascular growth (occurring in days - weeks) iv) sprouting angiogenesis microvessels (occurring in days).⁵¹ Mother vessels may retain their large bore size, acquire a smooth muscle and internal elastica, and evolve into medium-sized arteries and veins. Alternatively, the endothelium of a mother vessel may project cytoplasmic structures into the lumen that form transluminal bridges, eventually dividing the main channel into smaller separate well-differentiated channels, known as 'daughter' vessels. In other instances, the bridging central structure evolves into a disorganized glomeruloid vascular body. A third fate involves focal invagination of connective tissue pillars from within the mother vessel, forming longitudinal splitting of the main vessel into two smaller vessels, a process called intussusception.^{52,53} Vascular branching may be mediated

by fibroblast growth factor.⁵⁴ Finally, mother vessels may undergo endothelial cell sprouting, the best characterized process of tumor angiogenesis.⁵⁵ The remainder of this discussion will focus on the steps following sprouting angiogenesis.

Basement membrane dissolution

Sprouting angiogenesis requires the focal dissolution of the basement membrane surrounding mother vessels. Activated endothelial cells in the mother vessel secrete a number of proteolytic enzymes, including plasminogen activator and matrix metalloproteinases, enabling endothelial cells to exit the vessel ablumenally.⁵⁶

Endothelial cell proliferation

In contrast to the quiescent endothelium of mature, nonpathological blood vessels, activated angiogenic endothelial cells proliferate rapidly.^{55,57}

Endothelial cell migration

Proliferating endothelial cells migrate out of the mother vessel into the extracellular matrix towards the angiogenic stimulus.⁵⁸ Destabilization of the mother vessel architecture is important for vascular sprouting. In the presence of VEGF, angiopoietin-2 binds to the Tie-2 receptor, competitively displacing angiopoietin-1. This ligand-receptor interaction triggers a decoupling of endothelial cells, pericytes, smooth muscle cells and extracellular matrix in angiogenic regions.^{59,60} Angiogenic endothelial cells express adhesion molecules known as the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin that facilitate migration and vascular survival.^{61,62} At the sprouting tips of growing vessels, endothelial cells secrete matrix metalloproteinases that enable invasion.^{63,64}

Vascular tube formation

The formation of a lumen within an endothelial cell tubule requires interactions between cell-associated surface proteins and the extracellular matrix. Among the identified cell surface proteins are hybrid oligosaccharides, galectin-2, PECAM-1, and VE-cadherin.⁶⁵⁻⁶⁷ Creation of vascular lumens involves co-migration of three populations of endothelial cells as a single cord-like structure. An internal endothelial population is sheathed by a second cell population possessing numerous intracellular vacuoles. The internal population undergoes rapid apoptosis within 12 hours of formation. The vacuoles of the surrounding population fuse with the plasma membrane and are secreted. The net result is extensive remodeling of the center of a solid vascular cord into a lumen. A third endothelial population intersperses with the formed endothelial outer layer and expands the luminal circumference.⁶⁸ It has been suggested that tumors may also form vascular channels without the participation of endothelial cells, but the occurrence and explanation of this phenomenon remains controversial.^{69,70}

Arterial-venous differentiation

Vascular tubes differentiate into vascular loops, defining functional arterial and venous sides of the neovasculature. While little is known about this process, key insights have been derived from studies of vasculogenesis, or embryonic vascular development. Molecular cues on the afferent and efferent arms of differentiating vessels are provided by the ephrin-B2 transmembrane ligand (marking arterial endothelium) and its receptor, Eph-B4 (marking venous endothelium).^{71,72} Ephrin ligand-receptor interactions occur at the cell-cell juncture of arterial-venous anastomoses and along the length of a newly forming arterial vessel and an adjacent

vein.⁷³ In the nervous system, ephrin-Eph guides axonal growth, maintains boundaries between neuronal compartments, and prevents inappropriate nerve cell mixing. Analogously, ephrin-B2/Eph-B4 in angiogenesis is thought to guide patterned development of arterial and venous boundaries.⁷⁴

Vascular stabilization

Before blood flow begins, newly formed vessels are stabilized through the recruitment of smooth muscle cells and pericyte. These periendothelial cells are associated in varying degrees with virtually every portion of the vascular system. Binding of ephrin-Eph mediates signals between endothelial cells and mesenchymal cells.⁷² The angiopoietin family of molecules also plays a central role, where angiopoietin-1(Ang-1) binds to the Tie-2 receptor on angiogenic endothelium. Ang-1/Tie-2 leads to: 1) promotion of vascular tubule formation; 2) promotion of endothelial survival; and 3) secretion of PDGF and other chemokines that recruit smooth muscle cells and pericytes to the new vessel.⁷⁵⁻⁷⁷ Co-cultures of pericytes or smooth muscle cells with endothelial cells show that cell-cell contact between these two populations leads to the secretion of activated transforming growth factor-beta, an endogenous angiogenesis inhibitor.⁷⁸ Vascular stabilization thus also facilitates suppression of further angiogenesis. Angiopoietin-2 (Ang-2) is a competitive ligand for the Tie-2 receptor, whereby Ang-2/Tie-2 binding destabilizes vessels by uncoupling periendothelial cells from endothelial cells.⁶⁰ In the presence of VEGF, Angiopoietin-2 is permissive of angiogenesis. In the absence of VEGF, Ang-2/Tie-2 binding leads to endothelial cell apoptosis and vascular regression.⁷⁹

THERAPEUTIC TARGETING

The clinical development of antiangiogenic therapy for cancer is advancing rapidly. In 1988, interferon alfa2a was first used as an antiangiogenic drug to treat children with life-threatening hemangiomas, a nonmalignant vascular tumor.^{14,80} The first selective angiogenesis inhibitor, TNP-470, was launched into clinical trial in 1992 for Kaposi's sarcoma and cancer of the prostate and cervix. By the start of year 2000, more than 40 inhibitors had entered clinical trial, with more than one dozen in Phase III studies.⁸¹ A wide variety of therapeutic strategies have been devised, targeting one or more steps of the previously described angiogenic cascade (Table 2).

Classifications

The term 'antiangiogenic drug' is used to describe a diverse group of agents that affect newly growing blood vessels. The pharmacologic targeting of tumor blood vessels may be divided into three major categories: 1) true angiogenesis inhibitors; 2) vascular targeting agents; and 3) non-selective antiangiogenic agents. All three are presently in human clinical trials (Table 3).

True angiogenesis inhibitors halt only vascular sprouting and do not destroy pre-established blood vessels within a tumor. In experimental systems, true angiogenesis inhibitors generally slow tumor growth. Their action becomes manifest in several days to a week or more. Clinically, the expected effect of true angiogenesis inhibitors is disease stabilization rather than tumor regression, although individual cases of partial or complete response have been reported in cancer patients.^{82,83}

Vascular targeting agents destroy the pre-existing tumor vasculature. In animal studies, the effect of these agents is observable within hours.^{84,85} Acute endothelial cell death, tumor vessel thrombosis, and tumor mass hypoxia and necrosis result. Clinically, vascular targeting agents result in acute tumor pain.⁸⁶

Nonselective antiangiogenic agents exert antiproliferative, anti-invasive or cytotoxic effects on multiple cell types, including angiogenic endothelial cells.⁸⁷ Pure selection of endothelium is not achieved with these agents, although adjustment of drug dose, schedule, or delivery mode may produce marked anti-endothelial effects. Several conventional cytotoxic chemotherapeutic drugs have shown antiangiogenic effects when administered to mice at concentrations far below the established maximum tolerated doses.⁸⁸

Antiangiogenic targets

The elucidation of discrete steps in the angiogenic cascade enables the rational development of antiangiogenic drugs. Current drugs in clinical trials exploit several broad targeting strategies aimed at angiogenic blood vessels.

Growth factor antagonists

The antagonism of growth factor production, transport, or receptor binding is an upstream approach to antiangiogenic therapy. Several drugs, such as suramin, interferon alpha, and Angiozyme, suppress production of angiogenic growth factors.⁸⁹⁻⁹¹ Monoclonal antibodies and soluble receptors have been developed against VEGF.^{20,92,93} VEGF targeting may also promote endothelial apoptosis by suppressing the production of paracrine survival factors.^{47,48}

Endothelial cell signal transduction inhibition

Small molecule drugs have been developed to inhibit the endothelial signal transduction caused by specific growth factor-receptor binding. Both selective (against VEGF or PDGF) and non-selective agents (either VEGF/bFGF/PDGF) are in clinical trial. Preclinical studies suggest that some of these agents may be more potent against slowly growing tumors than against rapidly growing tumors, possibly reflecting a broader expression of different angiogenic factors in the latter.⁹⁴ Examples of such agents include SU5416, SU101, SU6668, and ZD4190.

Inhibitors of endothelial cell proliferation

A variety of antiangiogenic agents inhibit endothelial cell proliferation.⁹⁵ Selective endothelial inhibitors are desirable because pathological endothelium is localized while the normal vascular endothelium, outside of the female reproductive system, remains quiescent. Non-selective inhibitors may show more dose-dependent effects favoring inhibition endothelial proliferation. Examples of both types of agents include TNP-470, thalidomide, squalamine, and captopril.

Matrix metalloproteinases inhibition

Inhibition of matrix metalloproteinases (MMP) activity interferes with both endothelial and tumor cell invasion into the extracellular matrix at primary and metastatic sites. The family of known MMPs comprise at least 20 distinct enzymes, of which MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are closely associated with angiogenesis.^{63,96,97} Selective and non-selective MMP inhibitors are now in advanced clinical trial. Examples of these agents include Marimastat, AG3340 (Prinomastat), Col-3, Neovastat, and BMS275291.

Endothelial surface marker targeting

Markers associated with tumor vasculature represent attractive targets for drug development. Integrins are cell surface receptors selectively expressed on angiogenic endothelial cells.⁹⁸ Disruption of the $\alpha_v\beta_3$ integrin by monoclonal antibodies or cyclic peptides leads to activation of p53 and endothelial cell apoptosis.⁹⁹ Prostate-specific membrane antigen (PSMA) has also been identified as an angiogenic target.¹⁰⁰ Examples of drugs in clinical trial include Vitaxin (humanized LM609), EMD121974.

Suppression of endothelial progenitor cells

At least one antiangiogenic agent, angiostatin, appears to preferentially select endothelial progenitor cells, compared to mature endothelial cells.¹⁰¹

Lessons from Early Clinical Trials

As the development of antiangiogenic therapy matures, an analysis of emerging clinical trial lessons reveals important insights for guiding new basic research and optimizing biopharmaceutical efforts:

1. Animal studies do not directly translate to human studies

Preclinical studies of nearly every antiangiogenic agent in clinical trial have shown rapid and dramatic antitumor responses in mice bearing experimental tumors.¹⁰² As a general rule, these effects have not been recapitulated in Phase I and II trials of the same agents in human cancer patients. Genotypic and phenotypic differences may exist between experimental tumor lines and spontaneous tumors in patients. Additionally, cancer patients have co-morbidities, drug regimens, and other environmental and dietary variables that may influence the angiogenic response.¹⁰³ The development of optimal animal models for studying antiangiogenic agents in human disease is a major research goal.¹⁰⁴

2. Host responder characteristics remain poorly understood

Of the estimated more than 6,000 patients who have received antiangiogenic monotherapy in clinical trials, three distinct groups have emerged: non-responders; patients with stabilized disease; and less frequently, patients with partial or, rarely, complete tumor shrinkage.¹¹ The interindividual traits that contribute to the heterogeneous response is not understood. Recent data suggests that immunologic, bone marrow, and monocyte characteristics may influence patient response to angiogenesis modulators.¹⁰⁵⁻¹⁰⁷ A detailed understanding of host responder traits will enable improved clinical trial design, more precise matching of drug to disease, and enrollment of optimal patients for efficacy studies.

3. Combination therapy may enhance clinical outcome

A growing number of clinical trials combine an antiangiogenic agent with a cytotoxic agent or radiation.¹⁰⁸ The rationale comes from experimental work showing that reducing tumor vascularity paradoxically increases intratumoral penetration of chemotherapy agents.¹⁰⁹⁻¹¹¹ as well as tumor cell radiosensitivity.¹¹²

4. Conventional oncology trial strategies require modification

Most antiangiogenic agents are not directly cytotoxic to tumor cells. Accordingly, previous clinical trial strategies for cytotoxic chemotherapy drugs may not be appropriate. For example, Phase I studies of antiangiogenic therapy should seek the optimal biological dose (OBD) rather than maximal tolerated dose (MTD).¹¹³⁻¹¹⁵ Phase II studies may select disease stabilization or time to progression as a primary measure of success, rather than tumor regression.^{114,116} Suppression of minimal residual disease using monotherapy may be more effective than primary attack on large, established primary tumors. Chronic, lifetime therapy is envisioned with these agents, and long-term trials of suppression will be required. Modification and standardization of trial strategies in alignment with biological principles is necessary for optimizing efficiency in pharmaceutical development of this field.

5. Cytostatic agents that do not change tumor mass require new standards for monitoring therapeutic response and imaging

With antiangiogenic therapy, evaluation of tumor size alone is inadequate. Antiangiogenic agents exert their effects on the tumor vasculature, so efforts are underway to adapt imaging technologies to capture changes in the tumor vasculature and to identify surrogate markers reflecting the angiogenic burden. Some clinical trial protocols incorporate measurement of angiogenic growth factors in patient serum, urine and other body fluids, although clinical validation of these surrogate markers with respect to therapy has not yet been established.¹¹⁷ Serial tumor biopsies with histopathological staining and counting of tumor microvessels has been proposed, but this is invasive and impractical for the large numbers of patients required in late-stage clinical trials. A number of available imaging modalities (CT, MR, PET, ultrasound) are adaptable to focus on vascular features. Validation and standardization of monitoring techniques for antiangiogenic therapy is a major requirement of the field.¹¹⁸

IMAGING ANGIOGENESIS

The clinical monitoring of antiangiogenic therapy requires an imaging modality that is capable of detecting tumor vascularity and its changes with high sensitivity and specificity. Tumor blood flow, blood volume, vascular density, and metabolism are anatomically and functionally associated with tumor angiogenesis.¹¹⁹ The small size (< 100 μm) of microvessels precludes direct visualization by conventional angiography. Patients enrolled in cancer trials often have late-stage disease, with a heavy tumor burden. Such tumors will possess an extensive, established vascular supply. Angiogenesis imaging systems, therefore, must be able to accurately quantify small changes against a potentially large signal background. Furthermore, antiangiogenic therapy is envisioned to require lifelong treatments, so a non-invasive, cost-effective technique would be highly desirable.

Existing technologies and techniques

Since tumor size monitoring will remain an important clinical goal for oncologists, conventional cancer imaging modalities are being examined for their ability to capture parameters reflecting the tumor vasculature:

Computed tomography (CT)

CT imaging can be performed with contrast agents to define the intravascular compartment, including blood flow, blood volume, mean fluid transit time, and capillary permeability.¹²⁰ Functional CT techniques can delineate increases in tissue perfusion that may reflect malignancy, even when there is no gross anatomical abnormality present.¹²¹

Ultrasound

Ultrasound imaging can identify vascular features in tumors at different levels of resolution (40 - 200 micron diameter vessels), depending upon the technique employed. Contrast-enhanced ultrasound using an intravascular agent can generate an index of blood flow, blood volume, or vascularity within malignant tissue. Targeted imaging using ultrasound destruction of microbubbles may provide even further resolution of the tumor vascular tree.¹²² Color flow doppler has been used to characterize tumor xenografts in mice¹²³ and solid tumors in patients.^{122,124}

Magnetic resonance (MR)

MR imaging can define both blood volume and blood vessel permeability using dynamic enhancement of blood pool contrast agents. Gadolinium-DTPA can distinguish between normal (non-leaky) versus malignant (leaky) tissues, reflecting the hyperpermeable tumor vasculature.¹²⁵ Contrast uptake also correlates with microvessel density in experimental tumors.¹²⁶ Administration of an anti-VEGF monoclonal to experimental breast cancers in mice produces decreased vascular permeability that is detectable by MRI.¹²⁷

Positron Emission Tomography (PET)

PET imaging is used to evaluate tumor metabolism, as well as blood flow and volume. A number of radiotracers, such as H_2O^{15} , ^{11}CO , and ^{18}FDG , are available to characterize neoplastic tissue. Antiangiogenic agents should diminish blood flow and subsequently decrease tumor metabolism. PET scanning is currently being used by the National Cancer Institute to study the effects of antiangiogenic agents. Radiolabeled fluoromisonidazole (FMISO) has been used to quantitate hypoxia in the rat glioma by PET and may provide functional information about the results of antiangiogenic therapy.¹²⁸

Novel imaging strategies

Specific molecular features of the tumor vasculature may be exploited for imaging:

Targeting integrins

Angiogenic endothelial cells express adhesion molecules that possess the RGD-motif, known as the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins.¹²⁹ Monoclonal antibodies directed against the $\alpha_v\beta_3$ integrin (LM609) have been covalently bound to paramagnetic liposomes (PML) to create a targeted imaging system capable of imaging tumor angiogenesis in a VX2 rabbit carcinoma by magnetic resonance.¹³⁰ The PML may also carry a payload consisting of an antiangiogenic or cytotoxic drug, a radioisotope, or a signal-enhancing moiety, thereby enabling additional targeted delivery to and imaging of angiogenic endothelium and tumor. Another approach employs phage display libraries to detect tissue-specific endothelial cell markers against which homing peptides, linked to a therapeutic or signal-enhancing molecule, can be addressed.^{131,132}

Imaging endothelial cell apoptosis

A number of antiangiogenic agents have been shown to cause endothelial and tumor cell apoptosis. Markers of cell endothelial cell apoptosis, such as annexin V, may be adapted for radiolabeling and imaging of sites showing antiangiogenic and anti-tumor drug action.¹³³

Vascular stabilization/angiopoietins

The tumor vasculature, unlike healthy blood vessels, are heterogeneous, immature and lack architecture stability.^{134,135} Selective imaging of either angiopoietin-1 (required for vascular stabilization) or angiopoietin-2 (required for destabilization) may localize and evaluate the state of the tumor vasculature.

Infrared signature

Infrared sensing of highly angiogenic tumors growing in mice demonstrates temporal evolution of the thermal signature (Li WW et al, Unpublished data). Clinical investigations have shown thermal anomalies associated with breast tumors, although molecular correlation to tumor angiogenesis has not yet been shown.^{136,137} The availability of high-resolution military and aerospace-grade infrared sensors, coupled to sensitive endothelial molecular markers, now enables detailed study of the infrared and hyperspectral signature characteristics of tumor angiogenesis. Infrared assessment of tumor angiogenesis may provide a convenient, noninvasive imaging system to monitor antiangiogenic drug therapy.

SUMMARY

Rapidly accumulating knowledge of tumor angiogenesis is providing critical insights into the biology of cancer as well as new opportunities for clinical intervention and imaging. Antiangiogenic and anti-vascular agents represent a new approach to cancer therapy. Although some animal experiments show that tumor regression may occur following angiogenesis inhibition, clinical trials of the first wave of antiangiogenic agents suggest that disease stabilization, rather than cure, is a likely outcome in late-stage cancer patients. Combination therapies, treatment of earlier-stage disease, and an improved understanding of host-responder characteristics are likely to improve the clinical result.

Imaging the tumor vasculature itself is a critical goal for optimizing the development of antiangiogenic therapy. Conventional techniques (CT, MR, ultrasound, PET) that are ordinarily used to document tumor mass may be adapted to measure vascular parameters such as blood flow, blood volume, permeability, microvessel density, and tumor metabolism. Tumor vessels are architecturally heterogeneous, so blood flow is dynamic in some regions and stagnant in others. Conventional imaging may therefore offer only limited information regarding the tumor response to antiangiogenic therapy. The late stage of cancer diagnosis in many patients presents a large and pre-established population of tumor blood vessels from which to measure change after therapy, making high sensitivity of the imaging system essential.

Future approaches for imaging angiogenesis per se will likely exploit the molecular features of new blood vessel growth. Novel imaging targets include cell surface integrins, endothelial apoptosis, angiopoietins and infrared signature of angiogenesis. These new imaging modalities, combined with optimized trial design and more potent antiangiogenic agents will

create a robust platform for bringing antiangiogenic cancer therapy into standard oncology practice.

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TABLE 1. Known angiogenic growth factors

Angiogenin
Del-1 protein
Fibroblast growth factor: acidic (aFGF) and basic (bFGF)
Follistatin
Granulocyte colony-stimulating factor (G-CSF)
Hepatocyte growth factor (HGF)/scatter factor (SF)
HIV-Tat
Interleukin-3 (IL-3)
Interleukin-8 (IL-8)
Leptin
Midkine
Placental growth factor
Platelet-derived endothelial cell growth factor (PD-ECGF)
Platelet-derived growth factor (PDGF)
Pleiotrophin (PTN)
Proliferin
Transforming growth factor-alpha (TGF-alpha)
Transforming growth factor-beta (TGF-beta)
Tumor necrosis factor-alpha (TNF?)
Vascular endothelial growth factor (VEGF)/vascular permeability factor (VPF)

TABLE 2. Therapeutic targets in tumor angiogenesis

Growth factor antagonists

- Inhibition of angiogenic factor production
- Anti-growth factor ribozymes
- Soluble growth factor receptors
- Monoclonal antibodies directed against angiogenic factors

Endothelial cell signal transduction inhibition

- Receptor tyrosine kinase inhibition
- Protein kinase C inhibition

Inhibitors of endothelial cell proliferation

- Cell-cycle inhibitors

Matrix metalloproteinases inhibition

- Selective inhibitors of MMP-2, MMP-9
- Non-selective MMP inhibition

Endothelial surface marker targeting

- Anti-integrin antibodies or cyclic peptides

Endothelial cell subpopulation inhibitors

- Suppression of endothelial progenitor cells

Endothelial cell destruction

- Vascular targeting agents

TABLE 3. Classification & examples of agents in clinical trials that affect tumor vasculature

<u>Agent</u>	<u>Sponsor</u>
<u>True angiogenesis inhibitors</u>	
a) Specific inhibitors of angiogenic growth factors	
• Angiozyme	Ribozyme Pharmaceuticals
• Avicine	AVI Biopharma
• Suramin	NCI
• rhu MabVEGF	Genentech
b) Inhibitors of growth factor-receptor binding	
• IMC-1C11	ImClone
• IM862	Cytran
• PI-88	Progen Industries
c) Specific tyrosine kinase inhibitors	
• PTK787	Novartis
• SU5416	SUGEN
• SU6668	SUGEN
d) Anti-endothelial proliferative agents	
• TNP-470	TAP Pharmaceuticals
e) Anti-integrin agents	
• EMD121974	Merck KgaA
• Vitaxin	MedImmune
f) Inhibitors of angiogenic factor production	
• Octreotide	Novartis
g) Upregulators of angiogenesis inhibitors	
• ImmTher	Endorex
h) Unknown mechanism	
• Angiostatin	EntreMed
• Endostatin	EntreMed
<u>Vascular targeting agents</u>	
a) Anti-tubulin agents	
• Combretastatin A4 Prodrug	OXiGENE
b) Ion transport inhibitors	

- Squalamine
- c) Receptor-driven inducers of endothelial apoptosis
 - CM101

Magainin Pharmaceuticals

CarboMed

Nonselective antiangiogenic agents

a) Low-dose cytotoxic chemotherapy drugs

- Cyclophosphamide
- 5-Fluorouracil
- Methotrexate
- Vinblastine

b) Matrix metalloproteinase inhibitors

- BMS275291
- Captopril
- Col-3
- Marimastat
- Neovastat
- Prinomastat
- Solimastat

Bristol-Myers Squibb

Bristol-Myers Squibb

CollaGenex

British Biotech

Aeterna Laboratories

Agouron Pharmaceuticals

British Biotech

c) Anti-cytokine agents

- Thalidomide
- CC 4047
- CC 5013
- CC 7085
- CDC801

Celgene Corp.

Celgene Corp.

Celgene Corp.

Celgene Corp.

Celgene Corp.

d) Cox-2 inhibitors

- Celecoxib

GD Searle

e) Anti-tubulin agents

- Paclitaxel

Angiotech

f) Cell locomotion inhibitors

- Interferon alfa2a

Hoffman-LaRoche

g) Ion flux inhibitors

- Carboxyamidotriazole

NCI

h) Anti-mitochondrial agents

- Apra

Cell Therapeutics

i) Nonspecific tyrosine kinase inhibitors

- Flavopiridol
- Genistein

NCI

Amino A

j) Copper-lowering agents

- D-Penicillamine
- Tetrathiomolybdate

NCI

University of Michigan

k) Cell cycle inhibitors

- Ro 317453

Roche

Computed Tomography

Chairmen: Ting-Yim Lee, PhD
Kenneth A. Miles, MD

Panelists: Chusilp Charnsangavej, MD
Elliot Fishman, MD*
Karen Horton, MD
Fred Lee, Jr., MD

RECOMMENDATIONS

Functional CT techniques have been validated as tools for measurement of various physiological parameters within human tumors. However, further study is required to validate their use as markers for the efficacy of anti-angiogenesis therapy. Specifically, a correlation between histopathological features of angiogenesis (e.g. microvessel density, expression of vascular endothelial growth factors) and functional CT imaging parameters needs to be established for a range of tumors. Data should also be obtained to demonstrate the changes in functional CT parameters resulting from anti-angiogenesis therapy. Such data could be readily obtained by including functional CT into research protocols that currently use conventional CT to monitor the morphological effects of anti-angiogenesis treatments.

One important advantage of CT is that it can be used to study almost all tumors in the human body. The following is a guide to the application of functional CT in tumor imaging:

Primary Tumors

lung, pancreas, kidney, lymphoma:	<i>perfusion, blood volume, MTT</i>
liver:	<i>perfusion</i>
brain:	<i>capillary permeability</i>

Secondary Tumors

lung, mediastinum, abdomen,	
pelvis, superficial metastases:	<i>perfusion, blood volume, MTT</i>
liver:	<i>perfusion</i>

Specific recommendations in the development of CT functional imaging in angiogenesis:

1. Techniques should be developed so that more than one functional parametric map can be derived from a single study.
2. The optimization of contrast-to-noise ratio with respect to patient dose should be investigated more fully.
3. The expected heterogeneity of tumor physiology would argue strongly for the usage of multi-slice scanners so that at least 2 cm in the axial direction can be covered. For cases where more than 2 cm is required, additional study with a second injection of contrast agent is warranted. The second study can be delayed by as short as 10 minutes from the first study.

4. The strength, volumes and injection rates of contrast media need to be tailored to the analysis method employed. The Fick principle based method requires injection rates above 10 ml/s, whereas the deconvolution based method and model dependent methods, namely, the two-compartment Patlak model and the distributed parameter model, requires a lower injection rate around 3-4 ml/s.
5. The framing rate, which determines the patient dose if the technique parameters for each image remain the same, is dependent on the parametric maps as shown in Table 2.

Table 2.

Parametric Maps	Framing rate
Blood flow	1-3 s per image
Mean transit time	1-3 s per image
Blood volume	1-5 s per image
Capillary permeability	1-10 s per image

6. The development for clinical use of novel CT contrast agents with long vascular residence times or larger molecular weights would improve the measurement of permeability especially, but also of perfusion, blood volume and transit time.

Magnetic Resonance Imaging

Chairmen: Robert C. Brasch, MD
King CP Li, MD

Panelists: Janet E Husband, MRCP FRCR*
Mary T Keogan, MD
Michal Neeman, PhD
Anwar R Padhani, MRCP FRCR
David Shames, MD
Karl Turetschek, MD

RECOMMENDATIONS

1. For validation of any proposed imaging method hypothesized to assess tumor angiogenesis, strong adherence is urged for the use of established surrogates of angiogenesis, defined by an established technique. In this way, the relative merits of various imaging methods can be more easily compared and potential bias introduced by “new” or non-quantitative angiogenesis measures can be avoided.
2. A mechanism should be established for the sharing of raw data among established investigators in the MRI/angiogenesis field, from both preclinical and clinical studies, to be used for evaluation of alternative and potentially superior analysis methods and to more quickly fulfill the need for statistical significance.
3. Confirmatory studies should be encouraged/supported to reproduce published “positive” or “promising” results from other investigators.
4. Comparison studies are to be encouraged/supported to determine which MRI methods work best or best in combination and to refine MRI acquisition techniques. Questions to be answered include; Which contrast medium, or no contrast medium, works best? Pulse sequences? 2D vs 3D? Does arterial input function need to be monitored? What analysis method? Which kinetic model? How many data points are necessary? With what temporal resolution? Best methods to display information? etc.
5. There is an urgent need for preclinical testing of the different imaging approaches on the anti-angiogenic drugs entering clinical trials. To facilitate that, there should be a mechanism for making these drugs available for pre-clinical imaging studies.

Nuclear Imaging / Positron Emission Tomography

Chairmen: Francis G. Blankenberg, MD
William C. Eckelman, PhD
H. William Strauss, MD*
Michael J. Welch, PhD

Panelists: Abass Alavi, MD
Carolyn Anderson, PhD
Stephen Bacharach, PhD
Ronald G. Blasberg, MD
Michael M. Graham, MD, PhD
Wolfgang Weber, MD

CONCLUSION/RECOMMENDATIONS

A wide variety of radiopharmaceuticals are available to measure a number of parameters that are altered by the use of anti-angiogenic drugs. In a number of cases these tracers have been fully validated, the methods are highly sensitive to changes in the biochemical process, and the parameters can be extracted from a single image.

With few exceptions, the effect of anti-angiogenic drugs on parameters that can be measured by nuclear imaging has not been evaluated.

It is recommended that:

- techniques currently being used in ongoing clinical trials of anti-angiogenic drugs be studied in animal models to evaluate the changes induced by anti-angiogenic therapy. These parameters include flow, metabolism blood volume and permeability. Positron tomography has the strength that the radionuclides involved (^{18}F , ^{11}C , ^{13}N) are constituents of many drugs.
- anti-angiogenic drugs themselves be labeled with these radionuclides to directly study the pharmacokinetics of the drug.

At the same time new approaches to monitoring anti-angiogenic drugs including integrins, annexin V, hypoxia agents, proliferative indices, and various receptor ligands should be evaluated in animal models. At the present time the animal model used are wild type tumor cell lines. It is recommended that:

- genetically altered cell lines and transgenic animals for tumors known to undergo angiogenesis be developed.
- reported gene imaging strategies be applied to address specific molecular and cellular processes related to anti-angiogenesis. At the present time, flow metabolism and blood volume changes following anti-angiogenic therapy are being monitored. Companion studies that are possible to be included in ongoing clinical trials include; - the monitoring of permeability, monitoring of metabolism (FDG), monitoring of flow, monitoring of blood volume and co-registration with either MR or CT.

Nuclear imaging allows the quantification of many parameters believed to be important in anti-angiogenic therapy. These parameters must be validated in animal models and could be an important index of the efficacy of anti-angiogenic therapy.

Nuclear/PET Recommendations

- Look at currently used methods (flow, metabolism, blood volume, permeability) in ongoing clinical trials
- Label anti-angiogenic drugs with radionuclides to directly study pharmacokinetics of the drug
- Evaluate and validate new approaches to monitoring anti-angiogenic drugs
 - Integrins
 - Annexin V
 - Hypoxia agents
 - Proliferative indices
 - Receptors

Desirable Characteristics of Tracers

- Widely available
- Fully validated
- Highly sensitive to changes in the biochemical process
- Biochemical parameters can be extracted from a single scan

Validation Studies

- Established imaging strategies for flow, metabolism, permeability, blood volume
 - Response to therapy in animal models using imaging techniques (PET, SPECT)
- Evaluation and validation of new approaches in animal models
 - Currently used wild-type tumor cell lines and animal models
 - Develop genetically altered cells lines and transgenic animals
 - Apply reporter gene imaging strategies to address specific molecular and cellular processes related to angiogenesis
- Quantitation of biochemical parameters before and after therapy

Companion Studies to Ongoing Trials

- Monitoring of permeability
 - Ga-67/68 transferrin for PET and/or SPECT
 - Radiolabeled albumin (i.e. F-18, I-123, Tc-99m)
- Monitoring of metabolism (FDG)
- Monitoring of flow
 - i.e. O-15 labeled water, Tc-99m sestamibi, Tl-201
- Monitoring of blood volume
 - i.e. O-15-labeled CO, Tc-99m-labeled RBCs
- Co-registration with either MR or CT?

Ultrasound

Chairmen: Katherine W. Ferrara, PhD
Christopher R.B. Merritt, MD

Panelists: Peter Burns, PhD
Stuart Foster, PhD
Robert Mattrey, MD
Samuel Wickline, MD

RECOMMENDATIONS

Contrast Ultrasound

The contribution of ultrasound to the evaluation of tumor neovascularity at all stages of tumor development deserves further investigation. Because of its unique capability of evaluating both structure and function, ultrasound has the potential of providing effective, low cost, sequential monitoring of vascular changes associated with the development of malignant tumors and the response of tumors to treatment. Although pathologic studies of cancer demonstrate an increased number of small microvessels, these vessels are not selectively imaged with computed tomography, magnetic resonance or conventional ultrasound and Doppler. Consequently, the non-selective enhancement provided with these modalities results in limited cancer detection. In contrast to these conventional forms of vascular enhancement, intermittent ultrasound and contrast ultrasound in general present a unique opportunities to selectively enhance different levels of the microvasculature. These techniques should be developed further in the near future, and also considered for incorporation in clinical trials.

High frequency ultrasound

The ability of high frequency ultrasound to measure sub mm/s flow velocities in the microcirculation as well as its ability to detect changes in cell viability via modulation of the backscatter coefficient make it a unique tool for the assessment and monitoring of angiogenically active processes in tissue. This technology should be further developed and evaluated in mouse models to improve and validate its quantitative capabilities in regard to the monitoring of angiogenesis. In particular, emphasis should be placed on examination of vessel morphology, as this aspect of angiogenesis is not readily studied with other imaging methods. The results of this effort should then be translated to clinical studies of ocular melanoma, malignant melanoma, basal cell carcinoma, and Kaposi's sarcoma and their responses to anti-angiogenic treatment.

Doppler ultrasound

The contribution of Doppler ultrasound to the evaluation of tumor neovascularity at all stages of tumor development deserves further investigation. Current clinical results using ultrasound have produced mixed results. This is due, at least in part, to the lack of ultrasound instrumentation designed specifically to detect and display tumor neovascularity, which should be the subject of additional research.

Targeted imaging agents

The promise of targeted imaging is that earlier stages of cancer and the efficacy of anti-angiogenic therapies might be diagnosed based on detection of molecular epitopes. At this point, the feasibility of ultrasound detection of such agents has been demonstrated in vitro, and in vivo for thrombus and activated leukocytes, but not yet in tumors. The advantage of targeted ultrasound imaging, in comparison to other imaging modalities, is the sensitivity of ultrasound to detect a single small bubble. This area should be the subject of significant research efforts in the near future.